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ASCORBATE DEGRADATION IN TOMATO LEADS TO ACCUMULATION OF OXALATE, THREONATE AND OXALYL THREONATE

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Running title: Ascorbate degradation in tomato

Significance statement: Ascorbate is a key molecule for plant metabolism and a marker of fruit nutritional quality, but how it is degraded is poorly studied *in vivo*. Here we used radiolabeling to determine which degradation products accumulate in tomato leaves, to evaluate how the ascorbate pool size affects the degradation rate, and to test whether the degradation rate could be reduced by manipulating an enzyme involved in ascorbate recycling. We suggest that controlling ascorbate degradation might be a means to increase or stabilize ascorbate content.

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Abstract: Ascorbate content in plants is controlled by its synthesis from carbohydrates, recycling of the oxidized forms and degradation. Of these pathways, ascorbate degradation is the least studied and represents a lack of knowledge which could impair improvement of ascorbate content in fruits and vegetables as degradation is non-reversible and leads to a depletion of the ascorbate pool. The present study revealed the nature of degradation products using [^{14}C]ascorbate labelling in tomato, a model plant for fleshy fruits; oxalate and threonate are accumulated in leaves, as is oxalyl threonate. Carboxypentonates coming from diketogulonate degradation were detected in relatively insoluble (cell wall-rich) leaf material. No [^{14}C]tartaric acid was found in tomato leaves. Ascorbate degradation was stimulated by darkness, and the degradation rate was evaluated at 63% of the ascorbate pool per day, a percentage that was constant and independent of the initial ascorbate or dehydroascorbic acid concentration over periods of 24h or more. Furthermore, degradation could be partially affected by the ascorbate recycling pathway, as lines under-expressing monodehydroascorbate reductase showed a slight decrease in degradation product accumulation.

Keywords: ascorbate degradation, monodehydroascorbate reductase, light environment, tomato, [^{14}C]ascorbate labelling, high voltage paper electrophoresis.

L-Ascorbate (AsA) is a small organic acid derived from sugars. In plants, ascorbate is a key molecule involved in numerous cellular processes (cell division and expansion, photo-protection, enzyme cofactor, cell signalling; Smirnoff and Wheeler, 2000). Ascorbate is an essential antioxidant for plants, protecting the cell from reactive oxygen species (ROS). Curiously, ascorbate can also serve as a pro-oxidant, generating for example the highly reactive hydroxyl radical ($^{\bullet}OH$; Fry, 1998). The cellular concentration of ascorbate depends on its transport, biosynthesis, recycling and degradation: these are under genetic control and closely related to environmental conditions. According to multiple studies, one of the key genes of the biosynthetic pathway is *vtc2* (coding for GDP-L-galactose phosphorylase) which is up-regulated by light in leaves (Laing et al., 2015). Recycling of ascorbate's oxidation products (MDHA or dehydroascorbic acid (DHA)) to the reduced form (AsA) occurs via two enzymes monodehydroascorbate reductase (MDHAR) and DHA reductase (DHAR). MDHAR is an NAD(P)H-dependent enzyme which can reduce MDHA to AsA. DHAR uses glutathione as electron donor to reduce DHA to AsA. If not reduced by MDHAR or DHAR, MDHA and DHA may be further degraded, resulting in irreversible loss of ascorbate.

Ascorbate degradation in mammals, as in plants, has been poorly studied. In Figure 1, we sum up current knowledge about ascorbate degradation in mammals (vii and viii) and plants (i to vi). Degradation occurs *in vitro* via the unstable oxidized form DHA and can lead to the accumulation of end-products 2,3-diketo-L-gulonate, L-erythrulose (Nemet and Monnier, 2011), oxalate, L-threonate, L-xylosone (xylosulose), L-lyxonate, L-threosone and 3-deoxythreosone (Simpson and Ortwerth, 2000; Linster and Van Schaftingen, 2007). In humans, ascorbate degradation has been followed using radiolabelling experiments. After ingestion in the human body, 44% of the radioactivity from [1-^{14}C]ascorbate could be recovered in urine as oxalate, 20% as diketogulonate and 2% as DHA (Hellman and Burns, 1958). Recently, multiple studies have shown that ascorbate degradation products could interact with proteins and lead to the formation of advanced-glycation-end-products (AGE), suspected to play a role in complications in diabetes and cellular degeneration (Regulus et al., 2010; Kay et al., 2013). In *Escherichia coli* (Figure 1 ix), ascorbate can be used as source of carbon under anaerobic conditions, through enzymatic degradation to generate D-xylulose 5-phosphate, which can then participate in the pentose phosphate pathway (Yew and Gerlt, 2002).

In plants, the *in vivo* degradation pathway involves enzymatic reactions as well as non-enzymatic ones starting from ascorbate or DHA (Green and Fry, 2005a). End-products of the degradation pathway are species-dependent and include L-tartrate or oxalate and L-threonate (Figure 1; Green & Fry 2005b; Hancock & Viola 2005; DeBolt *et al.* 2006; Melino *et al.* 2009). Tartrate is mainly formed by cleavage of the ascorbate skeleton between carbons 4 and 5; in this pathway, the tartrate would thus be derived from carbons 1–4 of the ascorbate. The only

enzyme in this pathway identified for the moment is L-idonate dehydrogenase (DeBolt et al., 2006), which catalyzes the conversion of L-idonate (derived from ascorbate) into 5-keto-gluconic acid, a precursor of L-tartrate. This degradation pathway may occur enzymatically or non-enzymatically in species such as grape (grape berries are also known to accumulate oxalate (DeBolt et al., 2004), and thus use two degradation pathways within the same organ) and other *Vitaceae* (Hancock and Viola, 2005). Tartrate accumulation could also be the result of a conversion of L-threonate to L-tartrate, as in *Pelargonium crispum* (Wagner and Loewus, 1973), in which case the tartrate would be derived from carbons 3–6 of the ascorbate. In grape berries, tartaric acid deriving from ascorbate degradation is stored as calcium or potassium salts (DeBolt et al., 2004) during fruit development until 50 days after anthesis, but it appears that only a small proportion of the total ascorbate is actually used for the synthesis of L-tartrate (Melino et al., 2009). Tartaric acid is the major acid found in wine, contributing to its taste.

The formation of oxalate and threonate results from the cleavage of DHA between carbons 2 and 3. Intermediates between DHA and the oxalate and threonate end-products in the apoplast of *Rosa* cells (Green and Fry, 2005b) were initially proposed to be, sequentially, cyclic oxalyl L-threonates and oxalyl L-threonates. However, a kinetic study clarified that cyclic oxalyl L-threonates, oxalyl L-threonates and oxalate + threonate are all formed simultaneously, presumably from a highly reactive initial oxidation product of DHA, proposed to be cyclic-2,3-*O*-oxalyl-L-threonolactone (Parsons et al., 2011). This branched degradation pathway, observed in the apoplast and *in vitro*, might also occur in other cellular compartments. The proposed pathway could occur non-enzymatically *in vitro* but some steps are catalyzed by unknown enzymes as some reactions occur more rapidly *in vivo* (Green and Fry, 2005b). In lemon geranium, L-threonate is accumulated (Helsper and Loewus, 1982) but can also be decarboxylated to L-glycerate (Loewus, 1999). Oxalate accumulates in spinach, wood sorrel, shamrock and begonia (Yang and Loewus, 1975). In dock leaves (Helsper and Loewus, 1982), 24h radiolabelling experiments with [U-^{14}C]ascorbate revealed the following radioactive distribution: 1% in tartaric acid, 14% in threonic acid, 11% in oxalic acid, 14% in remaining ascorbate, 49% in others compounds and 11% in carbon dioxide (CO_2). When oxidation is minimised, DHA degradation occurs instead via hydrolysis into diketogulonate (DKG degradation branch; Fig. 1). Diketogulonate can be rearranged to form lactones provisionally identified as carboxypentonates, which can themselves be de-lactonised but are otherwise stable *in vivo* (Parsons et al., 2011); DKG can itself also be oxidised (Parsons and Fry, 2012).

There is little information about the potential role of these degradation products in plant cells. Oxalate is a very simple dicarboxylate which can be found in vacuoles (as also its soluble potassium, sodium or magnesium salts or insoluble calcium salts). Oxalate can be very abundant in oxalate-accumulating plants, reaching 3% up to 38% of dry mass (Libert and

Franceschi, 1987). Oxalate can be rapidly linked to calcium leading to the formation of calcium oxalate crystals, localized in vacuoles, cell walls (Khan, 1995) and specialized cells (idioblasts). Ascorbate degradation could be the first source of oxalate required for the formation of crystals with calcium in many species (Loewus, 1999; Franceschi and Nakata, 2005), with the exception of rice (Yu et al., 2010) and probably a few others species. Calcium oxalate crystals are found in a wide diversity of plants and animals. These crystals may be responsible for the regulation of the calcium pool of the cell (Nakata, 2003) but could also act in the defence process against herbivory (Franceschi and Nakata, 2005). Oxalate can be further oxidized in by oxalate oxidase (mostly reported in monocots) releasing H_2O_2 and CO_2 which may be used as an internal source for photosynthesis as oxalate oxidase is up-regulated by light (Loewus, 1999, Tooulakou et al., 2016). In species with no oxalate oxidase activity like in *Arabidopsis*, a cytoplasmic oxalyl-CoA synthetase may be required as a first step for oxalate degradation to CO_2 (and formic acid; Foster et al., 2012), as also previously described in pea, pumpkin and lupin seeds and wheat germ (Giovanelli, 1966).

Even if calcium oxalate crystal formation confers tissue rigidity and support, it has been revealed that calcium oxalate crystals are not necessary for growth processes in *Medicago truncatula* (Nakata and McConn, 2003; Nakata, 2012). Despite the remaining gaps in our knowledge of ascorbate catabolism pathways, the degradation rate and the expression of ascorbate oxidase are often positively correlated to cell expansion (Lin and Varner, 1991; Dumville and Fry, 2003; Müller et al., 2009), which is curious since ascorbate oxidase degrades ascorbate even though rapid growth usually correlates with high ascorbate concentrations.

Genetic and environmental impacts on ascorbate synthesis and recycling pathways have been well studied in a large diversity of plants (Hamner et al., 1945; Bartoli et al., 2006; Dowdle et al., 2007; Gautier et al., 2008; Massot et al., 2013). Light is proposed to be the most important environmental parameter altering ascorbate content. Ascorbate biosynthesis is activated by light: an increase of 66% in the ascorbate content in tomato leaves was found when plants were transferred from darkness to light (Hamner et al., 1945), although it was unclear whether this effect was due to increased biosynthesis or decreased degradation. Environmental control of the degradation rate has been poorly studied. Conklin et al. (1997) showed that radiolabelled ascorbate infiltrated into detached *Arabidopsis* leaves was rapidly oxidized in 24h in the dark. In contrast, in leaves of wood sorrel, degradation occurred at the same rate in light or darkness (Yang and Loewus, 1975).

Solanum lycopersicum is a model plant and one of the largest crops in the world. No information on either degradation products or the degradation rate is available in tomato. In this study, the aims were (i) to determine which degradation products accumulate in tomato

leaves; (ii) to evaluate the impact of ascorbate pool size on its degradation rate; (iii) to assay potential adjustment of the degradation rate by the activity of ascorbate recycling (by manipulation of MDHAR activity in transgenic plants). This study therefore aims to characterize ascorbate degradation products and the degradation rate under the influence of both environmental and genetic factors in tomato.

Materials and methods

Plant material and growth conditions:

Solanum lycopersicum L. variety West Virginia 106 (cherry tomato) cotyledons were transformed as previously described by (Gest et al., 2013). Plantlets overexpressing MDHAR were labelled sx lines in this paper. Plantlets underexpressing MDHAR were labelled mds lines in this paper. Wild type plantlets were used as reference (WT). Tomato plantlets used for radiolabelling experiments were grown in a greenhouse located at the University of Edinburgh, Scotland. Tomato plantlets used for GC-MS analysis were grown in climatic chambers located at INRA, Avignon. Tomato plantlets were acclimated for 2 weeks under short days (i.e. 8h light/16h dark) in low light conditions (100 $\mu mol.m^{-2}.s^{-1}$) and then a batch of plantlets was placed in high light conditions (1000 $\mu mol.m^{-2}.s^{-1}$) for 7 days while another batch stayed in low light for 7 days. A period of darkness for 48h was applied before final sampling to evaluate the effect of ascorbate pool size on ascorbate degradation rate.

Chemicals:

Solid L-[1-^{14}C]ascorbic acid (0.52 or 0.407 $MBq.\mu mol^{-1}$) from GE Healthcare was dissolved in water, aliquoted and stored at -80°C until required. [^{14}C]Mannitol (2.18 $MBq.\mu mol^{-1}$) was from Amersham International.

Incubation of tomato leaves with [^{14}C]ascorbate:

Tomato leaves were picked at 9am and quickly transferred such that the cut base of the petiole was in 5 kBq of [^{14}C]ascorbate diluted in 20µl of water. Pulse–chase incubation was performed. A constant air circulation was maintained during the whole incubation, and after approximately 1h (when the detached leaves had totally absorbed the radioactive solution), the [^{14}C]ascorbate solution was replaced by water. We followed radioactive ascorbate metabolism in a single leaflet kept under darkness for 24h and 48h. Sampling was performed as per the following time course: after 1 min of incubation, 30min, 1h, [add water], 2h, 3h, 4h, 6h, 8h and 24h. Tomato leaflets were immediately stored at -80°C before grinding.

Extraction of [^{14}C]ascorbate derivatives:

Tomato lamina samples were first weighed (50-60mg per leaflet) and then ground to a powder in liquid nitrogen. The powder was homogenized in 150µl of a solution containing 15% formic acid to extract ascorbate derivatives and 1.25% of non-radioactive ascorbate to prevent from further oxidation (the extraction process is summarized in Figure 2). One portion (10µl) of acid supernatant was analyzed by high-voltage paper electrophoresis (HVPE), and a further portion was assayed for total extracted radioactivity by scintillation counting. After quick rinses with 15% formic acid and water, the pellet was then treated with 2 ml of 1M sodium hydroxide (NaOH) at room temperature for 30 min to extract an additional fraction (including any polymer-linked esters). Half of the NaOH extract was scintillation counted, and half was dialysed against distilled water (2 x 12h) in Thermo Scientific dialysis tubes (mol. wt. cut-off 12,000) to remove small molecules while trapping polymers. After quick rinses with 1M NaOH and water, the tomato leaf residue was treated with 2M trifluoroacetic acid (TFA) at room temperature for 15min and centrifuged, then the TFA supernatant was volatilized to dryness using a SpeedVac, and extracts (10µl) were analyzed by HVPE. Radioactivity from all extracts was quantified by scintillation counting.

High-voltage paper electrophoresis (HVPE):

HVPE is particularly valuable for kinetic analyses of unstable radiolabelled organic acids such as ascorbate (Fry, 2011). Samples were dried on Whatman No.3 paper and electrophoresed in a volatile buffer at pH6.5 (acetic acid-pyridine-water, 1:33:300 by volume, containing 5mM EDTA) for 30min at 3.0kV or at pH2.0 (formic acid-acetic acid-water, 1:35:355 by volume) for 1h at 3.0kV. The papers were cooled to 20-25°C with toluene (for the pH6.5 buffer) or white spirit (for pH2.0) during the run. A trace of Orange G was loaded with the samples and used as an internal reference marker. Electrophoretic mobilities were reported as m_{OG} values (mobility, corrected for electro-endo-osmosis, relative to that of orange G). Authentic markers were purchased from Sigma Chemicals (Sigma) except for 2,3-diketogulonate, compound C and compound E which were eluted from previous experiments (isolated by elution from electrophoretograms in water). Non-radioactive compounds were stained with $AgNO_3$ or bromophenol blue (Fry, 2011).

Detection of radioactivity:

^{14}C-Labelled compounds on paper electrophoretograms were detected by autoradiography on Kodak BioMax MR-1 film. Alternatively, paper electrophoretograms were cut into strips and transferred into 23-ml Packard vials containing 2ml of Gold Star scintillant. Radioactive solutions were mixed with 10 volumes of 'OptiScint HighSafe'. ^{14}C was quantified by scintillation counting (LS 6500 Beckman; Beckman Coulter Ltd, High Wycombe, UK).

Ascorbate content:

Measurements of ascorbic acid content were carried out as previously described (Stevens et al., 2006). Ground powder was stored at -80°C then extracted in ice-cold 6% trichloroacetic acid (TCA). The spectrophotometric assay was based on the detection of dipyridyl-Fe^{2+} complexes following the reduction of Fe^{3+} to Fe^{2+} by ascorbate present in the sample. Total ascorbate plus dehydroascorbic acid content was measured by mixing the sample with 5mM dithiothreitol (DTT) to reduce DHA, prior to the assay. Each extract was measured in duplicate. The specificity of the assay has been checked by comparison with other known methods (Stevens et al., 2006) and by using ascorbate oxidase to remove all ascorbate in order to diminish other reductant background activity.

Extraction of ascorbate degradation compounds, derivatization and analysis using GC-MS:

Tomato leaves and fruit tissues were ground to powder in liquid nitrogen. Extraction was performed in 1000µl of previously degassed cold methanol with 120µl of internal standard ribitol (0.2mg.ml^{-1} in water). The mixture was extracted by mixing during 20 min at 4°C, and then mixed vigorously with 400µl of water. After centrifugation at 12000 rpm, the supernatant was reduced to dryness in a Speed-Vac. Samples were immediately analyzed or stored for a maximum of 48h at -80°C. Dry residues were re-dissolved in 60µl of 20mg.ml^{-1} methoxyamine in pyridine and derivatized for 90min at 37°C [carbonyl groups are transformed into the corresponding oximes (methoximation)]. 8µl of retention time standards (a mixture of dodecane, pentadecane, nonadecane, docosane) were added before trimethylsilylation. MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide; 120 µl) was then added and incubation was continued for a further 30min at 37°C for trimethylsilylation (to increase volatility). Samples were then loaded onto the AI 3000 autosampler. Sample volumes of 1µl were injected into the GC column using a hot needle technique. Analyses were carried out with a Trace GC Ultra-ISQ GC-MS system (Thermo France). Gas chromatography was performed on a Thermo TR-5MS column (20m length x 0.10mm inner diameter x 0.10µm film thickness). The injection temperature was set at 230°C and the ion source to 200°C. Helium was used as the carrier gas at a flow rate of 0.4ml.min^{-1}. The following temperature schedule was set: 3.70 min isothermal heating at 70°C, followed by a 7°C.min^{-1} ramp to reach 280°C and a second ramp of 30°C/min to 320°C for a final heating of 1 min. Mass spectra were recorded at 6.6 scans.s^{-1} with an *m*/*z* 50-650 scanning range. Mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al., 2005). Relative concentrations were determined by comparison with a ribitol internal standard.

Statistical analysis:

Data were submitted to a three-way analysis of variance (ANOVA) taking into account the effect of genotype, environment and time and their interactions (XLStat software Addinsoft, France). Significant differences between treatments were assessed by a Fisher's test ($p<0.05$).

Results

Nature of ascorbate degradation compounds in tomato leaves

As previous results have shown degradation at night (Conklin et al., 1997), a 24h time course of sampling in darkness was carried out to assay catabolism of [^{14}C]ascorbate in the leaflet laminae of detached tomato leaves. We used three extractants to detect and identify different classes of ascorbate-derived compounds; in each case, the nature and quantity of the radiolabelled compounds present in both the soluble and insoluble fractions were determined. The radioactivity recovered in each fraction as a percentage of the total radioactivity is shown in table 1. The small amount (<3%) of alkali-extractable material (extracted with NaOH after formic acid treatment) was separated by dialysis into two parts: high and low molecular weight (M_r). The proportion of alkali-extractable low-M_r material (potentially derived from polymer-esterified oxalate and related substances) remained stable during the time course. Acid-soluble radioactivity slightly decreased with time. Part of the radioactivity (up to 8.5% after 24h of incubation) was trapped in the formic acid- and sodium hydroxide-insoluble pellet. Tests with 2M TFA, an acid stronger than formic acid but whose volatility nevertheless facilitates its use before HVPE, were carried out on the acid- and alkali-insoluble pellet in order to solubilise calcium salts, notably [^{14}C]oxalate. After this extraction, and volatilization of TFA, a white deposit was observed and HVPE of this deposit showed that no spot could be detected at the origin point (Figure 1a), where the hypothetical calcium oxalate should appear (as it is not mobile in HVPE). Two spots were detected and firstly labelled A and B (exhibiting an unusual U-shape) as their nature was not revealed by appropriate markers run alongside (Figure 1a). A and B were eluted, dried, mixed with orange G, and re-run at pH 6.5 before the paper electrophoretograms were cut into strips of 1cm and assayed for ^{14}C by scintillation counting (Figure 1b). Finally, the eluted sample of compound A was treated with NaOH and run at pH6.5 and it revealed its ability to inter-convert into two spots (Figure 3c). The strong hypothesis that A and B are respectively compounds C and E (as named by Green and Fry, 2005b) is supported by their mobility relative to the orange G marker and their ability to interconvert (which is a peculiarity of the previously characterized compounds C and E). Thus, the two spots were identified as carboxypentonates.

Characterization of the ascorbate-derived compounds in the formic acid-soluble fraction was performed by HVPE analysis at pH 6.5 and pH2.0. At pH 6.5 (Figure 4a) radioactive ascorbate derivatives are separated into: ascorbate and DKG (not clearly

separated), DHA, oxalyl threonate (OxT) and oxalate (OxA) predominantly, and small amounts of compound E and proposed to be 2-carboxy-L-xylonate; Parsons et al., 2011). At pH2.0 (Figure 5a), ascorbate and DHA are not appreciably separated but cyclic oxalyl threonate (cOxT), diketogulonate (DKG), compounds C and E, a mixture of oxalyl threonate isomers, and oxalate were identifiable. The intensity of the radioactive bands (pH 2.0 and pH 6.5) decreased for ascorbate, DHA and DKG over the time course while for oxalate, cyclic oxalyl threonate and oxalyl threonate the spot intensity increased.

The percentage of [^{14}C]ascorbate and [^{14}C]DHA decreased rapidly during the first 8h following incubation, giving evidence for the degradation of the ascorbate pool (Figure 4b). For the next 16h of darkness, [^{14}C]ascorbate and [^{14}C]DHA decreased more slowly to reach approximately 9% and 16% respectively of total radioactivity. Oxalyl threonates and oxalate (soluble in cold formic acid, thus *not* the calcium salt) are the two major compounds that increased during the time course. Oxalyl threonates and oxalate appeared simultaneously (as observed *in vitro*; Parsons et al., 2011) to reach 30% and 25% of total formic acid-soluble radioactivity respectively after 24h incubation. Compound E was also detected, and accumulated slightly during the first 8h of the experiment, then decreased.

At pH2.0, the percentage of [^{14}C]ascorbate + DHA (Figure 5b) constantly decreased during the time course from 70% to reach 30% of the soluble radioactivity. Oxalyl threonates, oxalate and cyclic oxalyl threonate appeared simultaneously and increased constantly to reach 8%, 30% and 10% respectively after 24h. DKG, which was not resolved from ascorbate on HVPE at pH 6.5, was clearly resolved at pH 2.0, and found to be abundantly present even at the earliest time-point (11%), thereafter diminishing (to reach 2% of the total radioactivity). Compounds C and E accumulated slightly during ascorbate metabolism (5% and 2% of total radioactivity after 24h).

Darkness activates degradation pathway independently of ascorbate pool size

To complement these radiolabelling experiments, a GC-MS survey of two major ascorbate degradation end-products, oxalate and threonate, was carried out on tomato leaves subjected to different light and dark conditions. Tomato plantlets were grown under high light conditions (1000 µmol.m^{-2}.s^{-1}) or low light conditions (100 µmol.m^{-2}.s^{-1}) for 7 days. A batch of tomato plantlets was removed and stored for future analysis. A second batch of tomato plantlets (from both conditions) was placed in darkness for 48h. Ascorbate and dehydroascorbic acid content were assayed by spectrophotometric analysis. The content of both was higher in high light plantlets than in low light plantlets (Table 2) after 7 days of the different light treatments. During darkness, AsA and DHA decreased whereas oxalate and threonate accumulated for plantlets previously grown under both low light and high light. The

ascorbic acid content decreased to 22–26% of its initial content (22% for initially low light plantlets, 26% for initially high light plantlets). The oxidation product (DHA) also decreased, to 30% (high light) to 35% (low light) of its initial content after 48 hours of darkness. Corresponding with the loss of both AsA and DHA, a 48-h dark period caused a substantial increase in the products of irreversible DHA oxidation: OxA (5.4- fold in the initially high- and low-light plants) and threonate (12.9- and 5-fold in the initially high- and low-light plants respectively).

Impact of a modification of MDHAR activity on degradation products

The impact of a modification of MDHAR activity on ascorbate degradation was evaluated using lines overexpressing or underexpressing MDHAR following the same HVPE protocol as described above. Under darkness, we observed a slight decrease in the accumulation of products from the DHA oxidation branch (i.e. oxalate and its esters) in MDHAR-silenced lines compared to overexpressing lines and WT (significantly decreased after 8h of incubation; Figure 6). No differences between lines were noticed for DHA hydrolysis products (i.e. DKG and its downstream products). Similar results were observed by GC-MS analysis on transgenic immature green fruits (Supplemental data Figure S1): OxA and threonate accumulated less in silenced lines than in WT and overexpressing lines. However, after 24h of metabolism, no difference between all genotypes could be detected (Table 3).

A constant degradation rate under darkness

Based on the accumulation of all degradation compounds and disappearance of [^{14}C]ascorbate and DHA (of all lines mixed), we evaluated that 63% of total radioactivity appeared in degradation products after 24h of darkness (Table 3). The degradation rate of ascorbate was evaluated at 2.6% per hour. However, the dynamics of the degradation rate might be not linear, as we observed that the first 8 hours of darkness are characterized by a steep slope compared with the slope between 8h and 24h of darkness (Figure 4 and 5). The degradation rate reported in 24h under darkness appears to be particularly constant during our experiments (rates calculated on 2 to 5 independent leaflets per genotype).

Discussion

Major oxidation products oxalate, threonate and oxalyl threonate are accumulated in tomato leaves in darkness

Characterization of soluble [1-^{14}C]ascorbate-derived compounds did not reveal any compound related to tartrate metabolism in tomato leaves; thus the ascorbate $\rightarrow$ threonate $\rightarrow$ tartrate pathway was not operative. Any tartrate formed via the L-idonate pathway ((i) in Figure 1) would not have included the ^{14}C of [1-^{14}C]ascorbate, and thus we would not have detected

348 it. However, we did detect DHA oxidation products: [^{14}C]oxalyl threonate, cyclic [^{14}C]oxalyl
349 threonate and [^{14}C]oxalate. For every [^{14}C]oxalate molecule formed, we can expect that one
350 non-radioactive threonate molecule is also produced during ascorbate degradation (via DHA),
351 following the stoichiometry of a 4-electron oxidation: [^{14}C]ascorbate $\rightarrow$ [^{14}C]oxalate + non-
352 radioactive threonate (Green and Fry, 2005b). Oxalyl threonate and cyclic oxalyl threonate are
353 alternative 4-electron oxidation products of ascorbate (Green & Fry 2005b; Parsons et al.,
354 2011). Oxalate and threonate are more stable, probably explaining why they are better-known
355 end-products of ascorbate degradation (Yang and Loewus, 1975). OxT and cOxT are
356 susceptible to enzymic hydrolysis, e.g. in plant cell-suspension cultures, by esterases which
357 are not yet fully characterised (Green & Fry, 2005b). In addition, there is evidence in plant cell
358 cultures for incompletely characterised enzymes that catalyse the oxidation of DHA (Green &
359 Fry, 2005b).

360 The DHA hydrolysis pathway initially yields DKG, which we detected transiently though
361 it later disappeared during the time-course. Low levels of compounds C and E, which are
362 proposed to be downstream carboxypentonates (2-carboxy-L-xylonolactone plus 2-carboxy-L-
363 lyxonolactone, and their common de-lactonisation product, respectively) arising from DKG
364 non-oxidatively (Parsons et al., 2011), were also detected in the formic acid extracts.
365 Production of such carboxypentonates *in vivo* is suggested to be highly dependent on
366 ascorbate concentration as residual ascorbate inhibits compound C formation but also on the
367 presence of H_2O_2 as highly oxidizing conditions divert DKG to oxidative pathways (Parsons et
368 al., 2011). Compounds C and E may not be metabolized further and appear to be quite stable
369 end-products of ascorbate degradation.

370 Up to 8.5% of the total radioactivity (after 24h in darkness) was inextractable by
371 consecutive treatments with formic acid and NaOH, but releasable from the NaOH-insoluble
372 fraction by TFA. Surprisingly, this radioactive material comprised mainly the
373 carboxypentonates (C and E) mentioned above (Figure 3). Their presence in the alkali-
374 insoluble fraction of tomato leaves is curious because compounds C and E are water-soluble
375 and would have been expected to be rapidly released by aqueous formic acid. Further
376 analyses could be carried out to investigate their potential biological role, as they represent up
377 to 8.5% of ascorbate labelling.

378 Some of the ascorbate degradation products from either DHA or DKG can contribute
379 to H_2O_2 release. This phenomenon can occur *in vivo* (Kärkönen and Fry, 2006). Furthermore,
380 oxalate could be enzymatically degraded (Foster et al., 2012), leading to form CO_2. In addition
381 to the compounds cited above, we suggest that H_2O_2 and CO_2 may have been released during
382 the experiment (but not measured).

Decreasing MDHAR activity lowers the DHA oxidation rate

It is worth noting that ascorbate recycling ensures a relatively high turnover rate of the ascorbate pool, estimated at approximatively 13% per hour in pea seedlings (Pallanca and Smirnoff, 2000). However, Haroldsen et al. (2011) showed that, in tomato fruits, DHAR and MDHAR overexpression led to a depletion of the ascorbate pool. Similarly, the transgenic lines studied in this paper overexpressing MDHAR also show a decrease in ascorbate levels in leaves, and the silenced lines show an increase in ascorbate content both in leaves and fruits (Gest et al., 2013), with no or slight effect on the DHA concentration. The activity of the isoform 3 of MDHAR, targeted in this study, is therefore negatively correlated to vitamin C (AsA + DHA) content in tomato leaves, which is surprising since ascorbate is a product of MDHAR. These results are not explained by changes in the expression of genes of the biosynthetic pathway, or by changes in the activity of other enzymes involved in ascorbate recycling (DHAR and glutathione reductase). The rate of DHA oxidation in these transgenic lines, reported in the present manuscript, may offer a possible explanation for this ascorbate phenotype. Lines silenced for MDHAR show a lower accumulation of irreversible degradation products (OxT, oxalate, threonate etc.) in our experiments in darkness, agreeing with the higher ascorbate content in leaves. Further experiments will be conducted in leaves and fruits to test if the DHA oxidation rate could be partially under the control of MDHAR recycling enzyme or the MDHA radical.

Ascorbate pool size does not affect the percentage degradation rate of ascorbate in tomato leaves

Smirnoff and Wheeler highlighted the D-mannose/L-galactose pathway as the main biosynthetic pathway of ascorbate in plants (Wheeler et al., 1998). The recycling pathway affects the redox state of the ascorbate pool and is especially important during stress responses (Noctor and Foyer, 1998). Light intensity and light quality are the prominent environmental factors influencing ascorbate biosynthesis and recycling (Li et al., 2009; Massot et al., 2012). The degradation rate of ascorbate in different light environments had never been studied before in tomato leaves. We found higher ascorbate content in plantlets kept under high light than those grown under low light in line with current observations on the activation of synthesis and recycling by light (Bartoli et al., 2006; Gautier et al., 2008). Oxalate and threonate increased during darkness but not during high light stress or low light treatment. In the light, oxalate and threonate do not accumulate but this does not mean that degradation does not take place in the light as oxalate oxidase may be light-promoted as mentioned above (Loewus, 1999). Ascorbate levels of high light and low light plantlets were not similar at the beginning of the darkness treatment but the quantity of degradation products was proportional to the size of the total ascorbate pool (representing 20% of the initial content after 48h). If we

consider the final concentration of the vitamin C pool (AsA+DHA) after 48h of treatment, it represents 56% of the initial content. The degradation rate under darkness could be evaluated at about 1.5% per hour. This result is comparable to those obtained by (Conklin et al., 1997) on *Arabidopsis* where 40% of ascorbate degraded in the dark during 24h in *Arabidopsis* detached leaves. However, if we consider the fact that GC-MS analysis does not allow the detection of oxalyl-threonate, cyclic-oxalyl-threonate, DKG and carboxypentonates (thus, supporting the choice of HVPE analysis), this result is clarified by those obtained using radiolabelled experiments, where we noticed a degradation rate of about 2.6% per hour. The fate of the abundant DKG, observed at 1 h (Figure 5a), is unclear; it is possible that some of the [1-^{14}C]DKG was oxidatively decarboxylated to $^{14}CO_2$ plus a non-radioactive C_5 product such as 2-keto-L-xylonate, as observed *in vitro* (Deutsch, 1998; Parsons and Fry, 2012). Products mostly coming from DHA oxidation accumulated in the dark compared to those coming from DKG (the product of DHA hydrolysis), which remained at 5 to 10 % of radioactivity measured following the first hour of incubation. These products did not accumulate in the soluble fraction during ascorbate degradation under darkness, but might be trapped into insoluble material as mentioned above.

These multiple experiments support the hypothesis that the percentage degradation rate in darkness is stable over 24h in tomato leaves, whatever the ascorbate content is at the beginning of the darkness period.

Conclusions

Ascorbate degradation in the light or dark was studied in tomato leaves. Oxalate, threonate and oxalyl threonate were identified as end-products of ascorbate degradation (DHA oxidation branch). No tartaric acid was detected. The degradation rate was evaluated at 63% after 24h in dark conditions, with a major part of the degradation coming from DHA oxidation rather than via DKG (DHA hydrolysis). Carboxypentonates were also detected and trapped through unidentified bonding in the insoluble (cell wall-rich) leaf material. Further analysis will be performed to highlight their potential biological role. The percentage degradation rate is independent of the initial ascorbate level over periods of 24h and is under environmental control. In order to understand how to increase or stabilize ascorbate content in plants and fruits, controlling the degradation rate could be a good solution as degradation of ascorbate is non-reversible, and control of MDHAR activity may be a solution to explore. Efficient ascorbate recycling will also enhance protection of the ascorbate pool from degradation.

Acknowledgements

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590 precursor for oxalate biosynthesis in rice. *J. Exp. Bot.*, **61**, 1625–1634.

591 **Table 1**: Distribution of ^{14}C in detached tomato leaves between formic acid- and NaOH-
592 extractable pools and the NaOH-inextractable residue after incubation with [1-^{14}C]ascorbate in
593 darkness. Detached leaves were incubated for 60min with the cut petiole in radioactive solution
594 then the leaves were transferred such that the petiole was in water for the next 24h. Results
595 are expressed as percentage of the total radioactivity detected in the tomato lamina. Acid
596 extraction included 15% formic acid homogenization followed by centrifugation. Basic
597 extraction was performed by adding 1M NaOH to the pellet after acid extraction. 'Soluble
598 polymers' were detected in the alkali extract by dialysis. NaOH-inextractable radioactivity was
599 solubilised in cold 2M TFA of the NaOH-resistant pellet.

	% of total radioactivity after			
	1h	**4h**	**6h**	**24h**
$SC_{(1)}$ **Acid extraction**	95	93	92	88
$SC_{(2)}$ **Basic extraction**	2	3	3	3
$SC_{(3)}$ **NaOH-soluble polymers**	0.02	0.04	0.05	0.10
$SC_{(4)}$ **NaOH-inextractable radioactivity**	3	4	5	9
$SC_{(5)}$ **Pellet**	≈0	≈0	≈0	≈0

600

Table 2: Ascorbate (AsA), dehydroascorbic acid (DHA) and their oxidative end-products oxalate (OxA) and threonate (ThrO) in tomato leaves grown under high light (1000 µmol.m^{-2}.s^{-1}) or low light (100 µmol.m^{-2}.s^{-1}), and after 48h of subsequent complete darkness. OxA and ThrO were assayed by GC-MS analysis and expressed in arbitrary unit.gFw^{-1}. Ascorbate and DHA content were assayed by spectrophotometric analysis and expressed in mg.100gFw^{-1}. Six replicates (independent plantlets) per condition were used. Different letters indicate significant differences ($p<0.05$).

	mg.100gFw^{-1}				*arbitrary unit.gFw^{-1}*		*arbitrary unit.gFw^{-1}*	
	AsA	p<0.05	**DHA**	p<0.05	**OxA**	p<0.05	**ThrO**	p<0.05
high light	127.9	*a*	26.7	*a*	8.9	*c*	1.3	*b*
48h dark	33.1	*b*	8.0	*b*	48.0	*a*	16.8	*a*
low light	41.8	*b*	8.5	*b*	5.7	*c*	0.5	*b*
48h dark	9.0	*c*	3.0	*c*	30.6	*b*	2.5	*b*

609 **Table 3**: Repartition of the radioactivity after 24h of [^{14}C]ascorbate metabolism in darkness in
610 different transgenic lines modified for their MDHAR activity. Lines under-expressing MDHAR
611 are mds42 and mds5, overexpressing lines are sx1.7 and sx6.10. Wild type (WT) is used as
612 reference. The Table shows scintillation counts of total soluble radioactivity in degradation
613 products and in ascorbate and dehydroascorbic acid pool. Results (mean ± standard deviation)
614 are expressed as percentage of total soluble radioactivity. 5 replicates for WT, sx6.10 and
615 mds42; 2 replicates for sx1.7 and mds5 lines were used. No significant difference ($p<0.1$) was
616 detected between genotypes.

	% of 14C recovered in AsA+DHA after 24h	p<0.1	% of 14C recovered in degradation products after 24h	p<0.1
WT	37 ±6.2	a	63 ±9.9	a
mds42	37 ±11.7	a	63 ±18.7	a
mds5	43 ±2.8	a	57 ±2.8	a
sx1.7	30 ±3.3	a	70 ±3.3	a
sx6.10	41 ±4.2	a	59 ±1.5	a

617

Figure legends

Figure 1: Ascorbate degradation pathways identified in (i) Vitaceae (tartaric acid pathway), (ii) Geraniaceae and cultured *Rosa* cells (DHA oxidative pathway), (iii) cultured *Rosa* cells (DHA hydrolysis pathway), (iv) *Pelargonium crispum*; (vii) mammals, (viii) human lens and (ix) bacteria. (v) has been reported mostly in monocots; (vi) in *Arabidopsis*, pea, pumpkin and lupin seeds and wheat germ. Enzymes except L-IdnDH (L-idonate dehydrogenase), AO (ascorbate oxidase), APX (ascorbate peroxidase), MDHAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), oxalate oxidase, oxalyl-CoA synthetase and esterase from oxalate pathway and those of the bacteria degradation pathway (not indicated here) are not required or are yet unidentified. Adapted from (i) DeBolt et al., 2004, 2006; Melino et al., 2009 (ii) Green & Fry, 2005b; Parsons et al., 2011 (iii) Parsons et Fry, 2012 (iv) Wagner and Loewus, 1973 (v) Loewus, 1999 (vi) Foster et al., 2012 (vii) Linster and Van Schaftingen, 2007; Simpson and Ortwerth, 2000, Nemet and Monnier, 2011 (viii) Nemet and Monnier, 2011 and (ix) Yew and Gerlt, 2002.

Figure 2: Flow chart of the methodology used to localize radioactivity within the leaflet lamina tissue. HVPE is for high voltage paper electrophoresis and SC is for scintillation counting. Each extraction was conducted as described in materials and methods. Results of $SC_{(1, 2, 3, 4, 5)}$ are shown in Table 1.

Figure 3: (a) Accumulation of alkali-inextractable [1-^{14}C]ascorbate derivatives in tomato leaves under darkness. After acidic and basic extraction as described in figure 2, samples of the TFA-solubilised, NaOH-insoluble material were dried on Whatman paper No.3 and electrophoresed in a volatile buffer at pH6.5 (acetic acid-pyridine-water, 1:33:300 by volume, containing 5mM EDTA) for 30min at 3.0kV. Spots on the autoradiogram were not identified by comparison with non-radioactive markers run alongside. (b) A and B were eluted, dried, mixed with Orange G, and re-run at pH 6.5 (as described in (a)) before paper electrophoretograms were cut into strips of 1cm and assayed for ^{14}C by scintillation counting. (c) Eluted fraction of compound A was treated with NaOH and ran at pH6.5 (as described in (a)). Streaks were compared to compound A untreated.
Compounds A and B are believed to be lactonised (compound C) and de-lactonised (compound E) carboxypentonates respectively (Parsons et al., 2011).

Figure 4: Fate of [^{14}C]ascorbate in wild-type tomato leaves in the dark: analysis by HVPE at pH 6.5. (a) After a 1-h pulse of [1-^{14}C]ascorbate fed to tomato leaves via the cut petiole, followed by a chase of up to 24 h in non-radioactive water, metabolites were extracted from leaflets with formic acid and analysed by HVPE at pH 6.5. An autoradiograph is shown. Spots are identified by reference to stained markers.

(b) Quantification of the radioactive spots by scintillation counting. AsA, DHA and DKG-derivatives are shown as white symbols; downstream metabolites are shown as black symbols: oxalyl threonate (OxT) and oxalate (OxA). Results are expressed as percentage of total HCOOH-soluble radioactivity.

Figure 5: Fate of [^{14}C]ascorbate in wild-type tomato leaves in the dark: analysis by HVPE at pH 2.0.
Other experimental details as in Fig. 4. Ascorbate (AsA) and dehydroascorbic acid (DHA) are both essentially uncharged at pH 2, resulting in a single radioactive spot near the origin.

Figure 6: Accumulation of degradation products derived from [^{14}C]ascorbate (similar protocol to that of figure 3) in leaves of different transgenic lines modified for their MDHAR activity. The graph shows lines under-expressing MDHAR (mds; white symbols), overexpressing lines (sx; grey symbols), and wild type (WT; black symbols). Scintillation counts of total soluble radioactivity in products coming from DHA oxidation (OxT, cOxT, OxA; triangles) and via DHA hydrolysis (DKG, C, E; circles) during 24h of dark incubation with [1-^{14}C]ascorbate are shown. Results (mean ± standard deviation) are expressed as percentage of total formic acid-soluble radioactivity. Six replicates for sx and mds lines and 4 for WT line were used. One star indicates a significant difference from the wild type WT ($p<0.07$).

Supplemental data legends

Supplemental data Figure S1: Oxalate (OxA) and threonate (ThrO) content in green tomato fruits of transgenic lines modified for their MDHAR activity. mds3 and mds5 are two independent lines under-expressing MDHAR (hatched bars). sx1.7 and sx6.10 are two independent lines overexpressing MDHAR (grey bars). Wild type (WT) is used as reference (white bar). Oxalate (OxA) and threonate (ThrO) were assayed by GC-MS and expressed in arbitrary unit/gFw. Five replicates were used per genotype. Different letters indicate significant differences ($p<0.05$).

Fig. 1

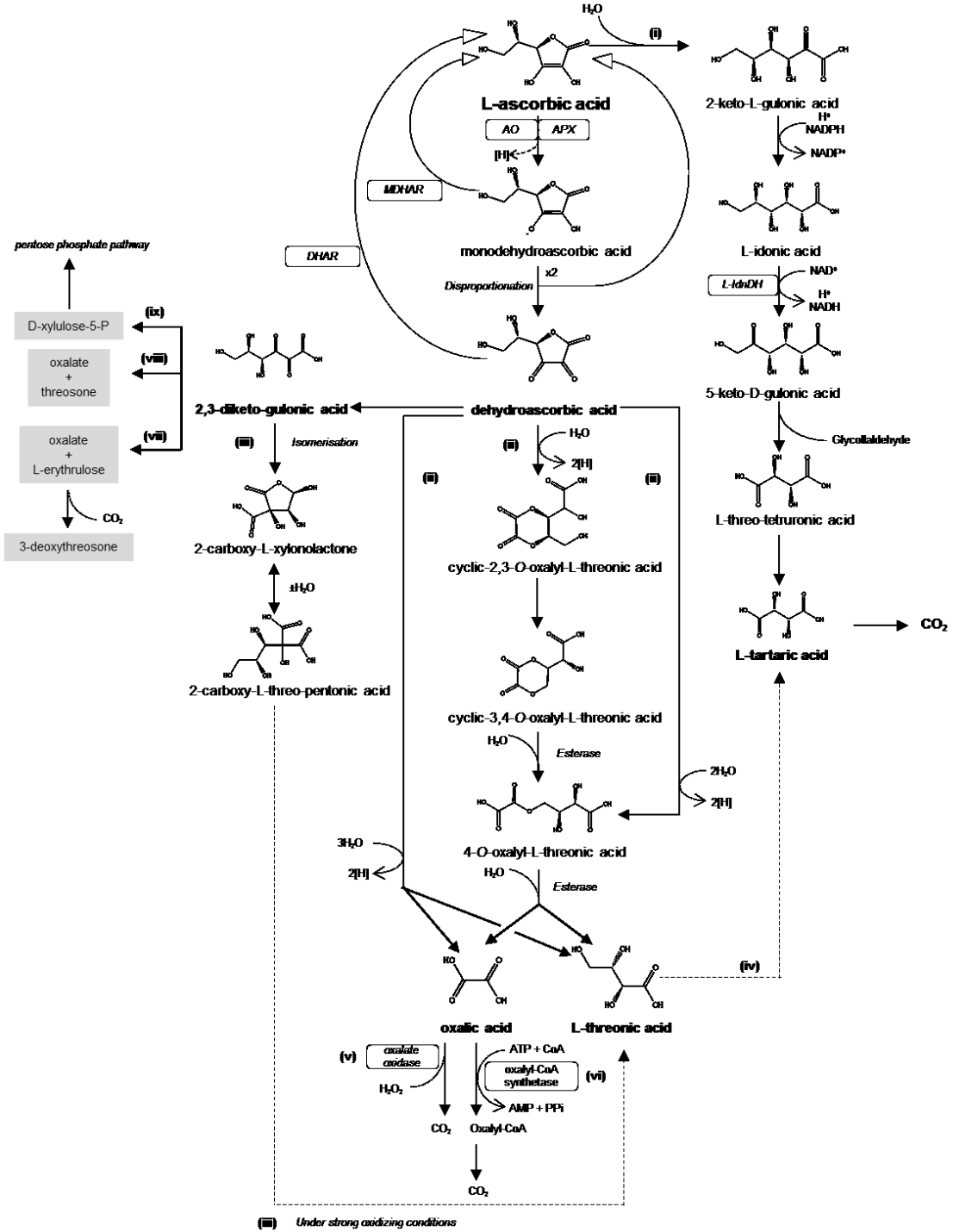

L-ascorbic acid
AO
APX
MDHAR
DHAR
monodehydroascorbic acid
Disproportionation
x2
dehydroascorbic acid
2-keto-L-gulonic acid
L-idonic acid
L-IdnDH
5-keto-D-gulonic acid
Glycollaldehyde
L-threo-tetruronic acid
L-tartaric acid
CO2
2,3-diketo-gulonic acid
Isomerisation
2-carboxy-L-xylonolactone
2-carboxy-L-threo-pentonic acid
cyclic-2,3-O-oxalyl-L-threonic acid
cyclic-3,4-O-oxalyl-L-threonic acid
4-O-oxalyl-L-threonic acid
Esterase
oxalic acid
L-threonic acid
oxalate oxidase
oxalyl-CoA synthetase
ATP + CoA
AMP + PPi
Oxalyl-CoA
pentose phosphate pathway
D-xylulose-5-P
oxalate + threosone
oxalate + L-erythrulose
3-deoxythreosone
Under strong oxidizing conditions

Fig. 2

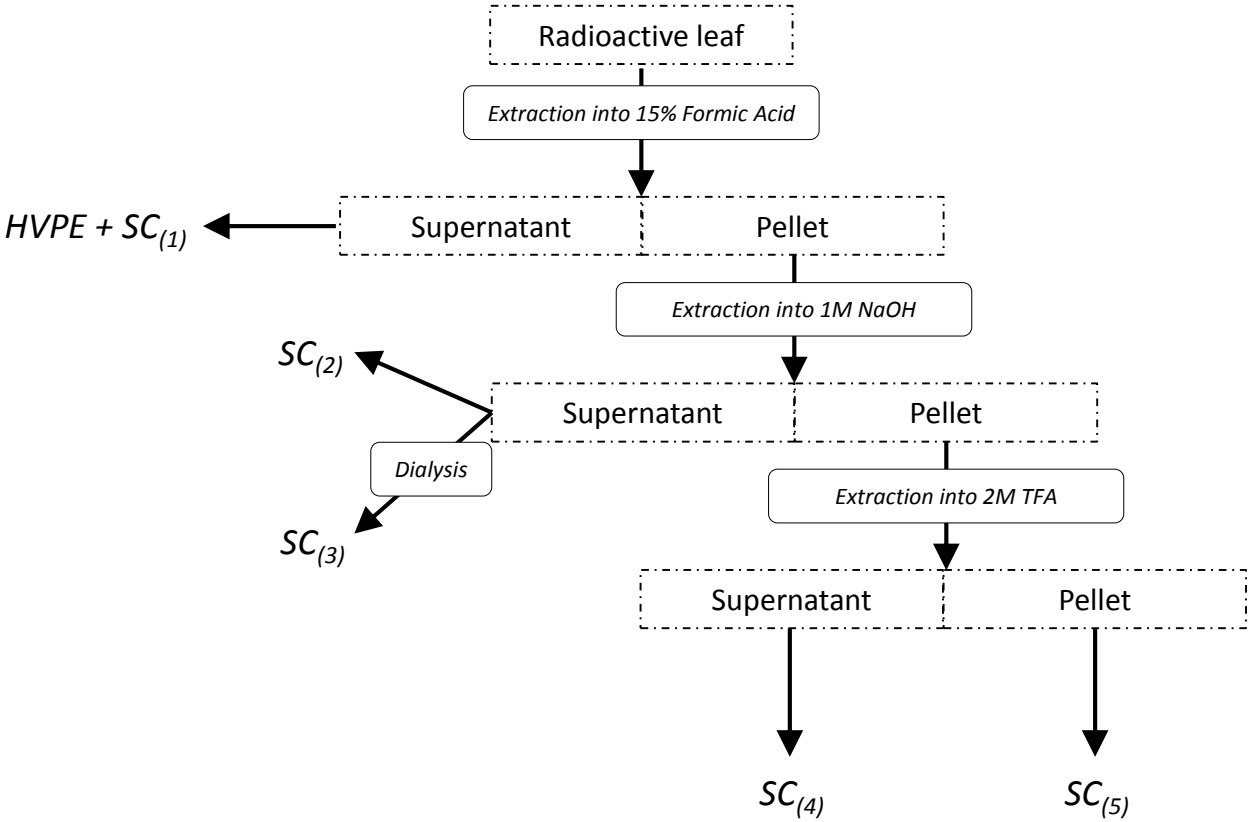
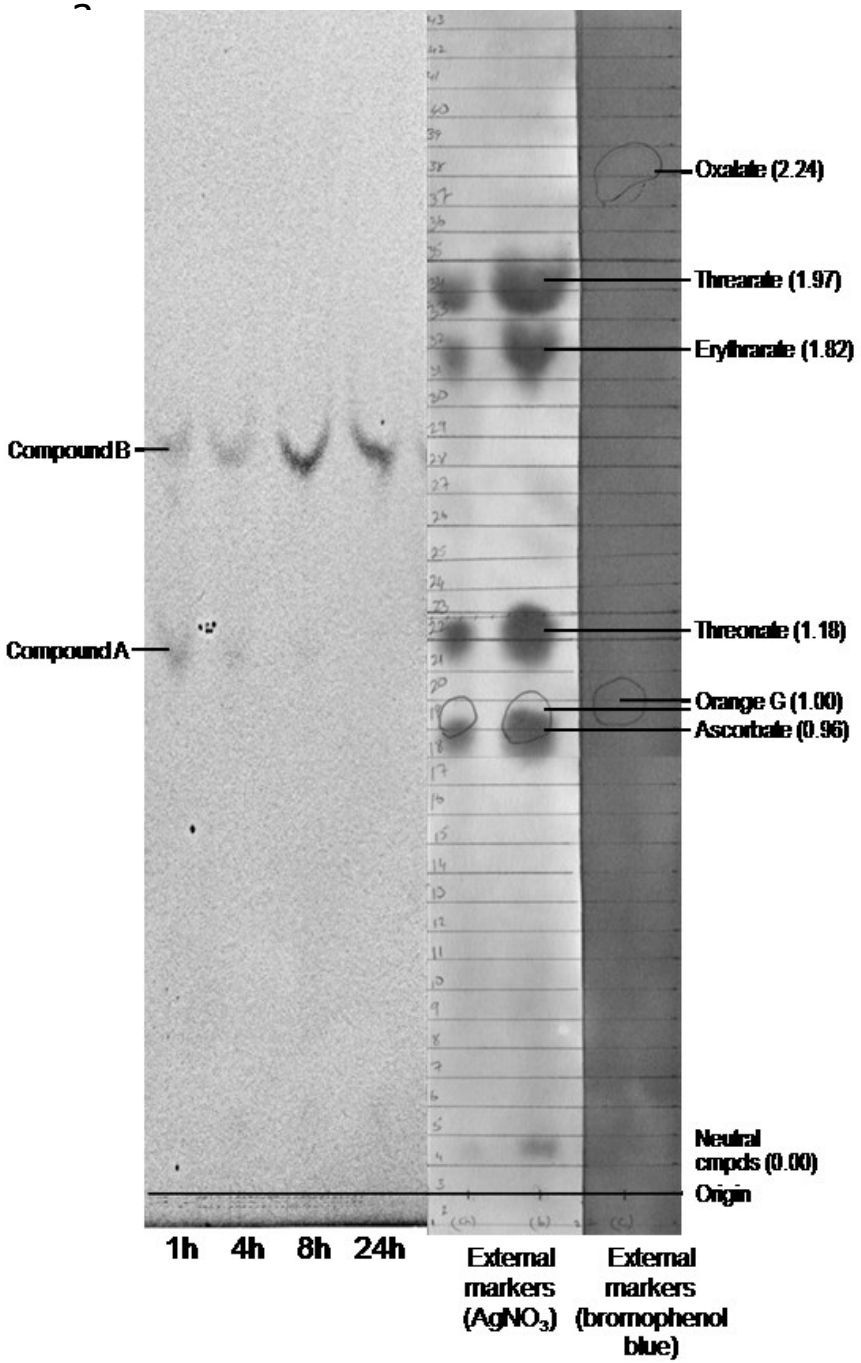
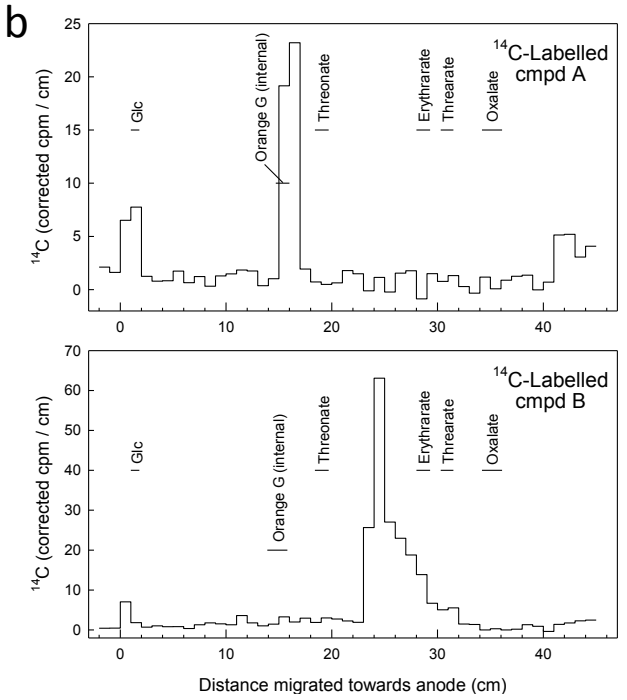

Radioactive leaf
Extraction into 15% Formic Acid
HVPE + SC(1)
Supernatant
Pellet
Extraction into 1M NaOH
SC(2)
Supernatant
Pellet
Dialysis
SC(3)
Extraction into 2M TFA
Supernatant
Pellet
SC(4)
SC(5)

Table 1

	% of total radioactivity after			
	1h	**4h**	**6h**	**24h**
$SC_{(1)}$ **Acid extraction**	95	93	92	88
$SC_{(2)}$ **Basic extraction**	2	3	3	3
$SC_{(3)}$ **NaOH-soluble polymers**	0.02	0.04	0.05	0.10
$SC_{(4)}$ **NaOH-inextractable radioactivity**	3	4	5	9
$SC_{(5)}$ **Pellet**	≈0	≈0	≈0	≈0

Fig. 3


Oxalate (2.24)
Threarate (1.97)
Erythrarate (1.82)
Compound B
Compound A
Threonate (1.18)
Orange G (1.00)
Ascorbate (0.96)
Neutral cmpds (0.00)
Origin
1h
4h
8h
24h
External markers (AgNO3)
External markers (bromophenol blue)


b
14C-Labelled cmpd A
14C-Labelled cmpd B
Glc
Orange G (internal)
Threonate
Erythrarate
Threarate
Oxalate
14C (corrected cpm / cm)
Distance migrated towards anode (cm)

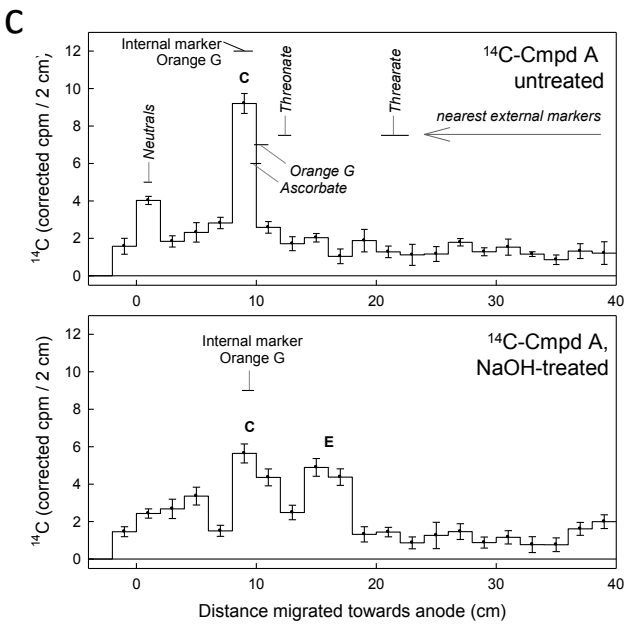
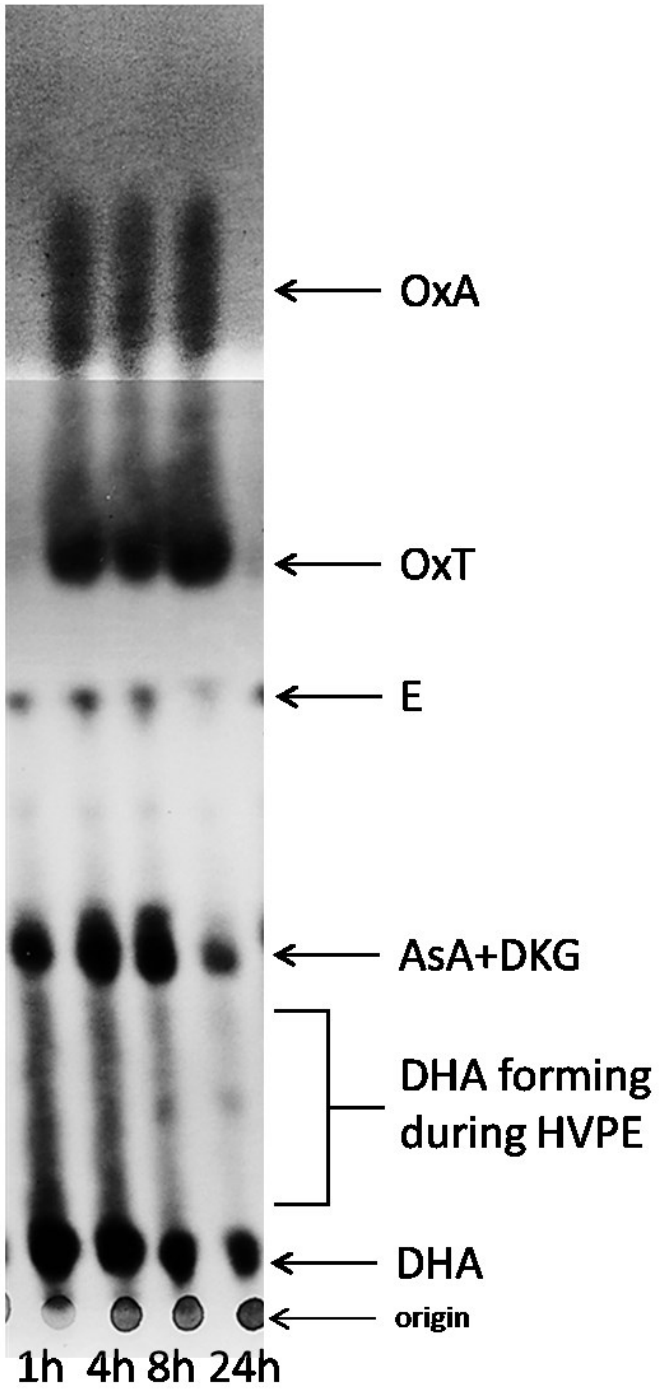

c
14C-Cmpd A untreated
14C-Cmpd A, NaOH-treated
Internal marker Orange G
Neutrals
C
Threonate
Threarate
nearest external markers
Orange G
Ascorbate
E
14C (corrected cpm / 2 cm)
Distance migrated towards anode (cm)

Fig. 4

a


OxA
OxT
E
AsA+DKG
DHA forming during HVPE
DHA
origin
1h 4h 8h 24h

b

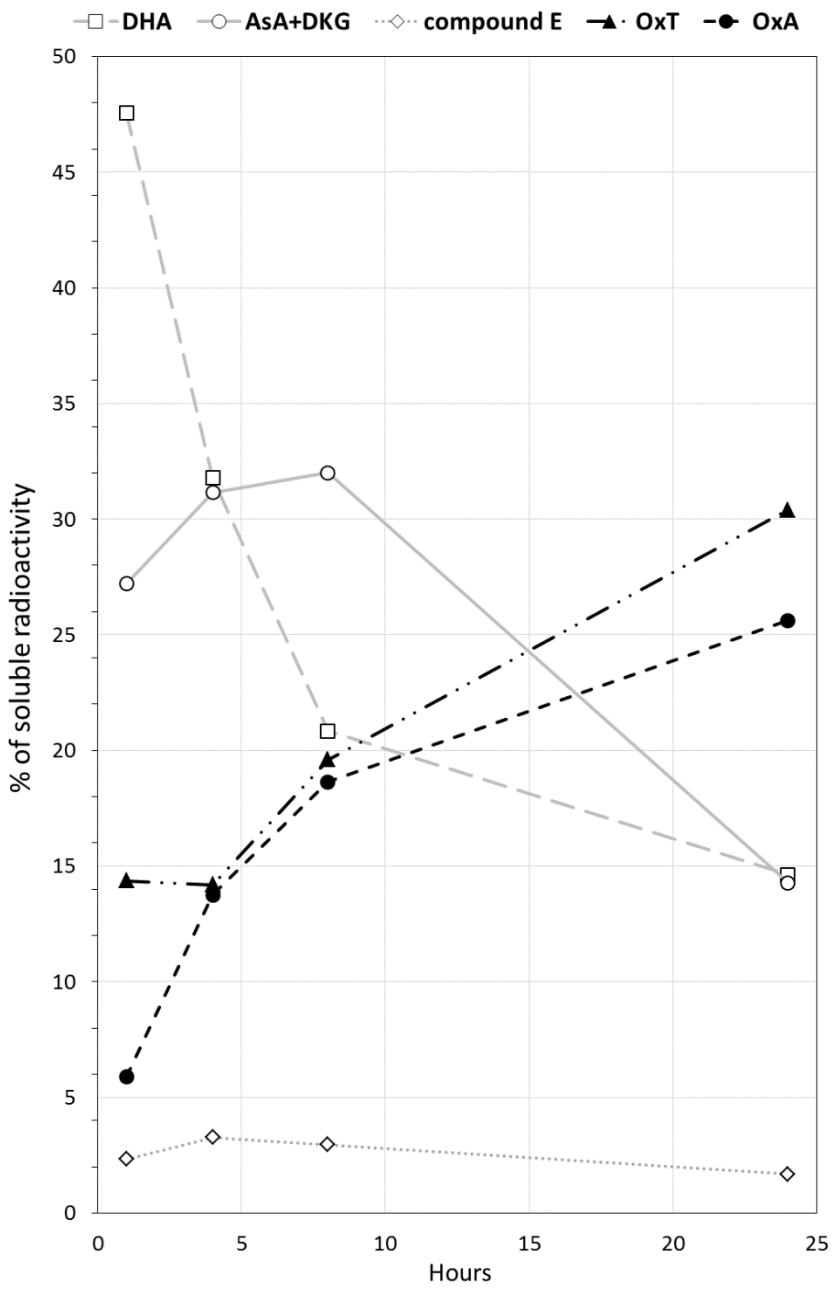
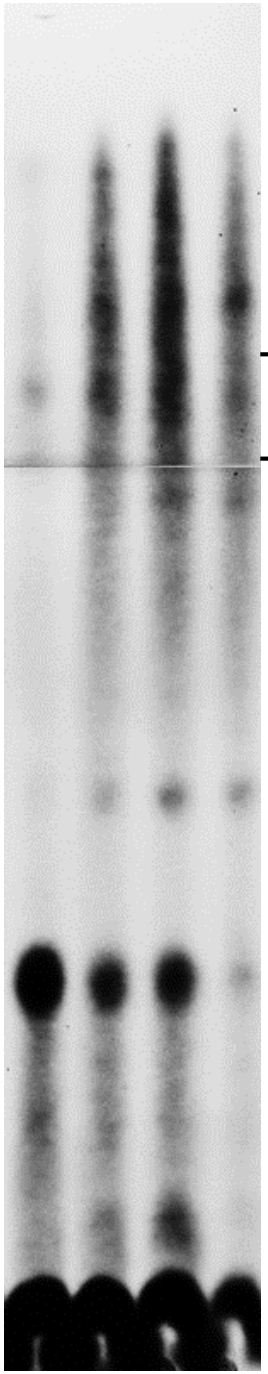

DHA
AsA+DKG
compound E
OxT
OxA
% of soluble radioactivity
Hours
0
5
10
15
20
25
30
35
40
45
50

Fig. 5

a


OxA
cOxT
Isomers of OxT
C
E
DKG
AsA + DHA
origin
1h 4h 8h 24h

b

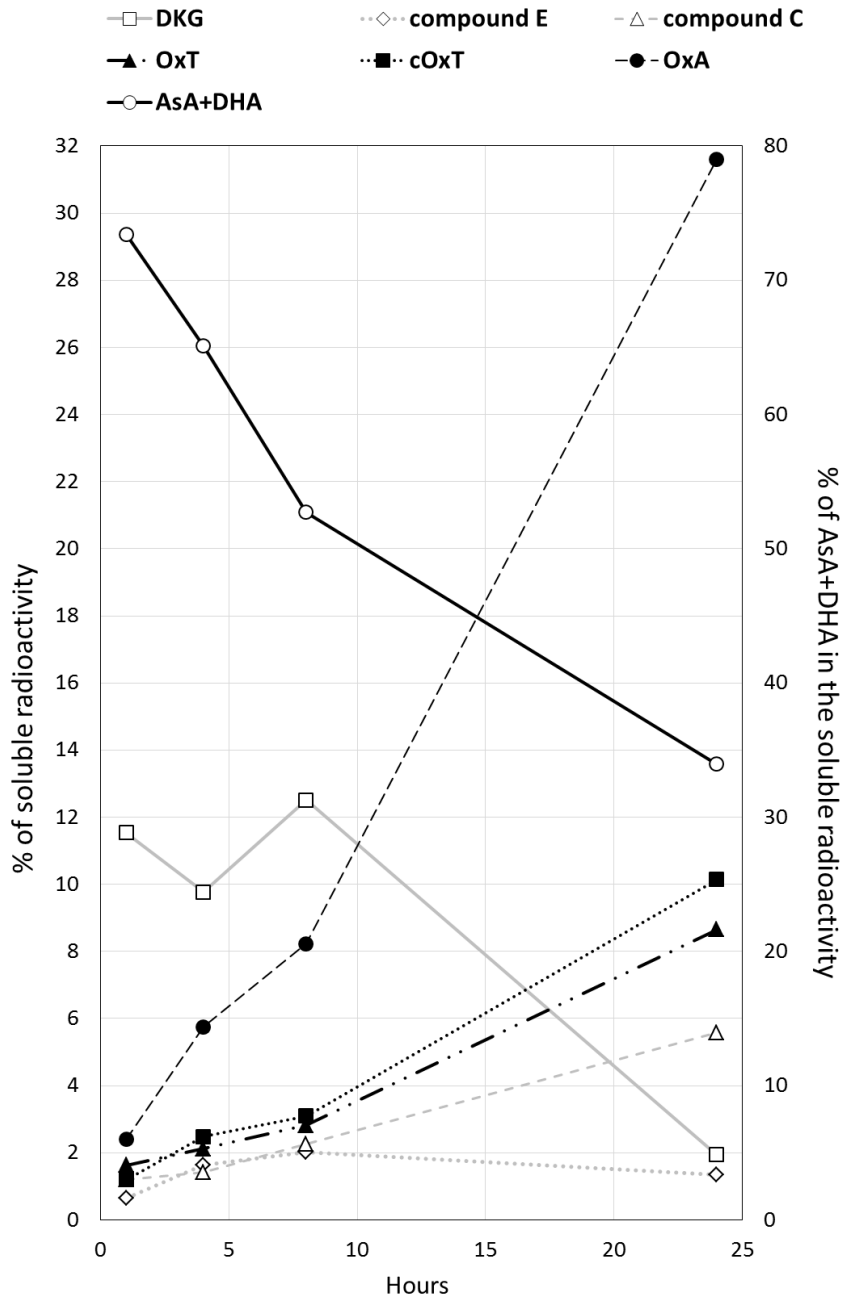

DKG
compound E
compound C
OxT
cOxT
OxA
AsA+DHA
% of soluble radioactivity
% of AsA+DHA in the soluble radioactivity
Hours
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25

Table 2

		mg.100gFw $^{-1}$			*arbitrary unit.gFw $^{-1}$*		*arbitrary unit.gFw $^{-1}$*	
	AsA	p<0.05	**DHA**	p<0.05	**OxA**	p<0.05	**ThrO**	p<0.05
high light	127.9	*a*	26.7	*a*	8.9	*c*	1.3	*b*
48h dark	33.1	*b*	8.0	*b*	48.0	*a*	16.8	*a*
low light	41.8	*b*	8.5	*b*	5.7	*c*	0.5	*b*
48h dark	9.0	*c*	3.0	*c*	30.6	*b*	2.5	*b*

Fig. 6

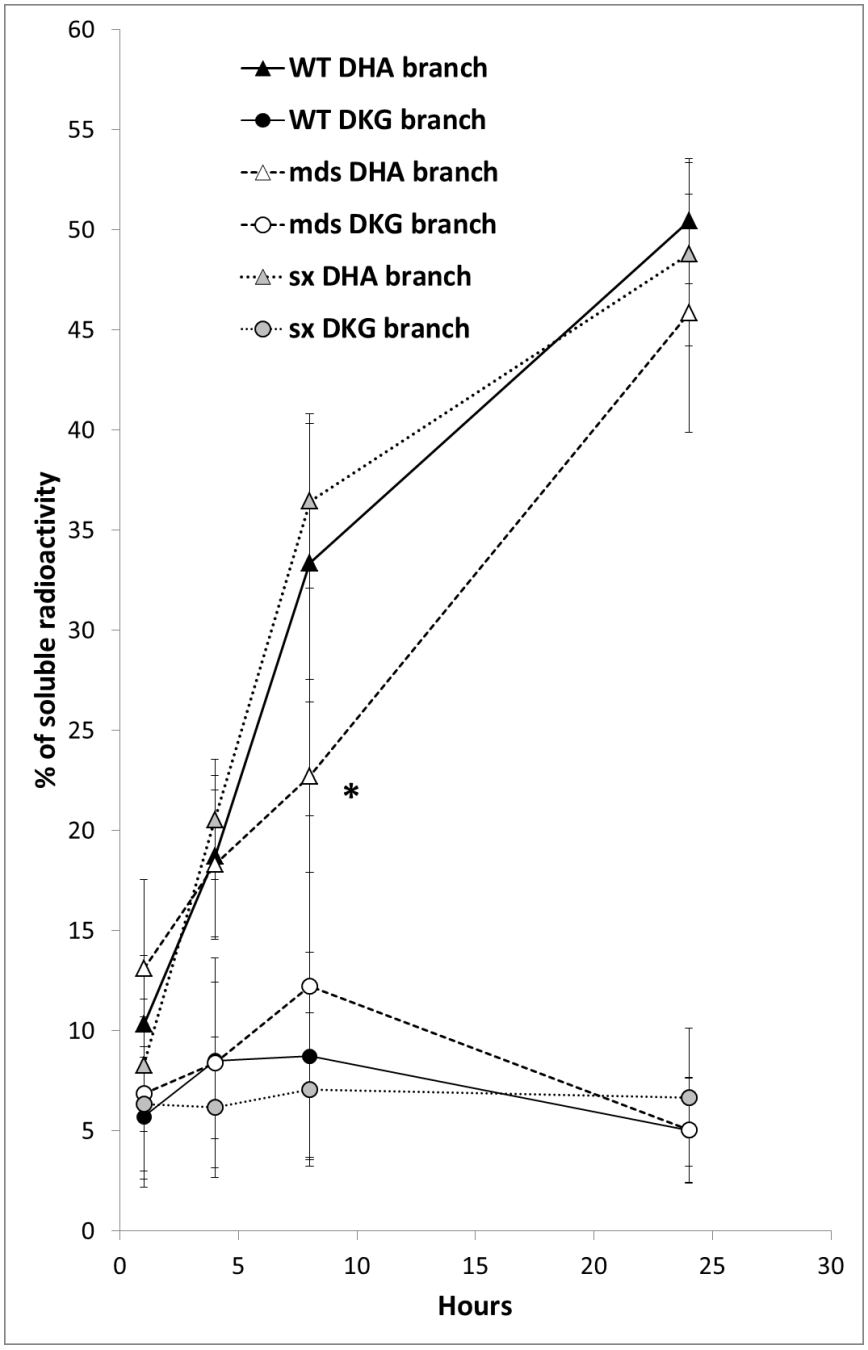
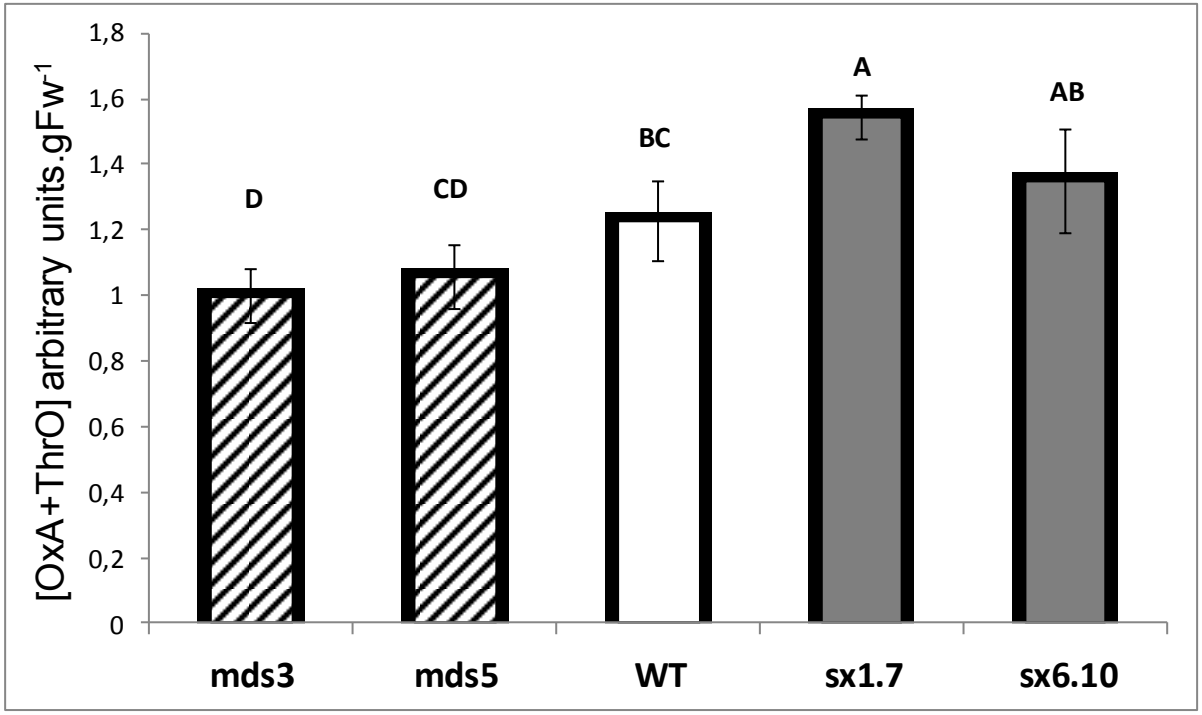

WT DHA branch
WT DKG branch
mds DHA branch
mds DKG branch
sx DHA branch
sx DKG branch
% of soluble radioactivity
Hours
*
0
5
10
15
20
25
30
35
40
45
50
55
60

Table 3

	% of 14C recovered in AsA+DHA after 24h	p<0.1	% of 14C recovered in degradation products after 24h	p<0.1
WT	37 ±6.2	a	63 ±9.9	a
mds42	37 ±11.7	a	63 ±18.7	a
mds5	43 ±2.8	a	57 ±2.8	a
sx1.7	30 ±3.3	a	70 ±3.3	a
sx6.10	41 ±4.2	a	59 ±1.5	a

Fig. S1


[OxA+ThrO] arbitrary units.gFw-1
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
D
CD
BC
A
AB
mds3
mds5
WT
sx1.7
sx6.10